Endogenous heme oxygenase induction is a critical mechanism attenuating apoptosis and restoring microvascular perfusion following limb ischemia/reperfusion

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Background. A protective role for endogenous heme oxygenase (HO) in the initiation of remote liver injury after limb ischemia/reperfusion has been established. This study expands on our previous work by investigating the role of endogenous HO on hepatocellular injury, hepatocyte death (necrotic and apoptotic), and microvascular perfusion at protracted post-reperfusion times.

Methods. Remote liver injury was studied after 1 hour of bilateral hind limb ischemia and 3, 6, or 24 hours of reperfusion in male C57BL6 mice. Inhibition of HO was achieved with the use of chromium mesoporphrin (CrMP). Established intravital videomicroscopy techniques were used to evaluate microvascular perfusion and hepatocyte death. Hepatocellular injury was quantified by serum alanine transaminase. Apoptosis was measured by using DNA laddering, Cell Death ELISA, and caspase-3 activity.

Results. Although significant perfusion deficits and hepatocellular injury/death occurred after 3 hours, progression of hepatocellular death beyond 6 hours was not observed. A transient increase in apoptosis was observed at 6 hours. By 24 hours, microvascular perfusion was completely restored. This lack of progression correlated with increased HO activity, observed throughout the protocol. Administration of CrMP reduced HO activity to sham nonstressed levels, and caused increased microvascular perfusion deficits, hepatocellular injury, and hepatocyte death over 24 hours. The transient increase in apoptosis was increased in duration and magnitude in CrMP-treated animals.

Conclusions. These results suggest that endogenous HO activity prevents the progression of remote liver injury after limb ischemia/reperfusion. (Surgery 2004;136:67-75.)

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Reperfusion of previously ischemic tissues or organs causes local injury and inflammation, but may also cause systemic inflammation resulting in injury to remote organs.1,2 Injury to the liver after remote I/R has often been overshadowed by concerns arising from respiratory, cardiac, and renal dysfunction. However, liver injury has been reported as a major contributor to mortality of patients suffering from multiple organ dysfunction after infrarenal aortic reconstruction.3,4 While mechanical and pharmacologic supports exist for other organs, it is not possible to support the failing liver, a condition that is often asymptomatic until >90% of the liver is damaged.

Liver injury after ischemia and reperfusion (I/R) occurs in 2 mechanistically distinct phases: the initiating phase of injury is neutrophil independent and is mediated by cytokines, coagulopathy, and Kupffer cell activation; the progressive phase is mediated primarily by neutrophils.5-7 Our laboratory has placed extensive efforts both in the characterization of the initiating phase of liver injury after bilateral hind limb ischemia/reperfusion and in the investigation of the enzyme heme oxygenase induction as a critical mechanism attenuating apoptosis and restoring microvascular perfusion following limb ischemia/reperfusion.
Heme oxygenase (HO) as an endogenous protective mechanism at these early time points. Heme oxygenase is the rate-limiting enzyme in the degradation of heme to carbon monoxide (a vasodilator), iron, which is sequestered by ferritin (an antioxidant), and biliverdin, which is converted into bilirubin (an antioxidant). Heme oxygenase is a heat shock protein (HSP32) and is highly inducible by a variety of stimuli, such as heat shock, ischemia, radiation, hypoxia, hyperoxia, inflammation, and disease states.

In our model of remote liver injury after bilateral hind limb I/R, endogenous HO activity does not provide a benefit to hepatic microvascular perfusion during the first 1.5 hours after reperfusion. However, endogenous HO does limit hepatocellular death in the pericentral regions (only) of the liver and reduces leukocyte–endothelial cell interactions at this early time point. These studies suggested an increasingly important role for endogenous HO activity at later postreperfusion times (3 hours), at which time HO limited microvascular perfusion deficits and hepatocellular death in both periportal and pericentral regions of the liver. In spite of the protection afforded by HO, its endogenous activity was insufficient to prevent injury during these early/initiating time points. Further study during the progression of remote liver injury over extended periods of time would be necessary before exploitation of HO activity could be considered in the development of therapeutic strategies.

In the present study, we build on our previous results by considering protracted post reperfusion times and demonstrate that remote liver injury does not progress beyond 6 hours after bilateral hind limb I/R. However, the administration of a selective competitive inhibitor for HO resulted in the progression of remote liver injury through 24 hours. These observations advance previous understanding of remote liver injury and further elucidate the role of HO in liver protection and recovery after limb ischemia/reperfusion. In addition, we further expanded the scope of our investigations by exploring the temporal nature of hepatic cell death via apoptosis and by investigating the modulation of apoptosis as a mechanism through which HO confers protection.

MATERIAL AND METHODS

Animals. Procedures were conducted in accordance with the criteria outlined by the Canadian Council on Animal Care and approved by the University of Western Ontario Council on Animal Care. Male C57BL/6 mice (weighing 23-27 g) were randomly assigned to 1 of 6 experimental groups, with a minimum of n = 5 per group. A systemic inflammatory response was initiated by 1 hour of bilateral hind limb ischemia followed by reperfusion. Measurements were made after 3, 6, or 24 hours of reperfusion.

Surgical procedure. Mice from all experimental groups were anesthetized by inhalation of isoflurane (5% induction; 2% surgery and maintenance) with a mixture of nitrogen (2.5 L/min) and oxygen (1 L/min). The left carotid artery was canulated (PE10 tubing) for continuous monitoring of mean arterial pressures and fluid resuscitation (0.4 mL/h normal saline). Ischemia was achieved by application of a tourniquet above the greater trochanter of each hind limb and standardized with the use of a force meter. After 1 hour of ischemia, the tourniquets were removed to allow reperfusion. Throughout surgery and ischemia, normal body temperature was maintained (36.0°C-37.0°C) with the use of heat lamps, while the level of fluid resuscitation was sufficient to maintain normal mean arterial pressure (80 mmHg-120 mmHg) throughout each experiment.

Inhibition of heme oxygenase. Chromium mesoporphrin (CrMP; Porphrin Products Inc, Logan, Utah) is a very selective competitive inhibitor of HO activity. At appropriate doses, CrMP is known to have no inhibitory effects on nitric oxide synthase or soluble guanylyl cyclase. Chromium mesoporphrin was administered via intraperitoneal injection immediately after onset of ischemia. The dose of 10 μmol/kg body weight was chosen for effective inhibition of HO to levels consistent with sham.

Intravital video microscopy. After reperfusion, animals underwent intravital video microscopy (sham animals underwent microscopy immediately after arterial cannulation). Mice were reanesthetized and a transverse incision was made across the midline just below the xiphoid. The left lobe of the liver was exposed and reflected onto the stage of an inverted microscope (Nikon Eclipse TE300; Nikon Canada, Mississauga, Ontario, Canada), moistened by a saline bathing solution containing the fluorescent vital dye propidium iodide (20 μg/mL; Sigma, St. Louis, Mo), and covered with plastic film to prevent dehydration and to minimize movement due to respiration. The liver was transilluminated by using a fiber optic light guide to provide sufficient contrast for microscopy. Random views of the microcirculation near the surface of the liver were observed on a video monitor with the use of a 20x objective lens. One-minute observations of 18
to 22 fields of view (equally divided among periportal and pericentral functional regions) were recorded for later analysis of sinusoidal perfusion and cell death. Immediately after intravitral video microscopy, a blood sample was obtained, and the liver was removed and stored at −80°C for later analysis.

Sinusoidal perfusion. Sinusoidal perfusion was evaluated by using established stereologic techniques. Briefly, a point-counting grid was chosen to obtain >95% confidence that the density of points counted was proportional to the density of sinusoids in the area of interest. Perfusion in a sinusoid was evaluated at every point on the grid that landed within the dimensions of a sinusoid. Perfusion in a sinusoid was then classified as continuously, intermittently, or non–red blood cell perfused. A continuously perfused sinusoid was one in which red blood cell perfusion was continuous throughout the 1-minute observation time. An intermittently perfused sinusoid possessed red blood cell perfusion, which stopped at least once during the observation period. A nonperfused sinusoid was either one devoid of red blood cells or one in which red blood cells did not move for the duration of the observation period. The number of sinusoids in each category was expressed as a percent of the total number of sinusoids evaluated.

Hepatocellular injury. At euthanasia, a blood sample was obtained by arterial exsanguination. Serum levels of alanine transaminase (ALT) were determined by standard enzymatic techniques. ALT is an enzyme found predominantly in hepatocytes and is released into the serum when the hepatocellular membrane integrity is compromised. Increased levels of serum ALT indicate wide-spectrum hepatocellular injury (mild through severe).

Hepatocellular death—in vivo labeling. Propidium iodide is a fluorescent vital dye known to stain only the nuclei of cells that are lethally damaged (via necrosis and end-stage apoptosis). Lethally damaged hepatocytes were labeled in vivo by direct application of propidium iodide (20 μg/mL in normal saline; Sigma, St Louis, Mo) and visualized with epiluminescence (excitation 510-560 nm; emission filter >590 nm). Stained hepatocyte nuclei were easily identified by their morphologic differences from other liver cell types. Hepatocellular death was expressed as the number of propidium iodide–labeled hepatocyte nuclei per square meter surface area of tissue.

Apoptosis—analysis of genomic DNA fragmentation. Genomic DNA from the liver was isolated according to our method in a previous study. Briefly, frozen liver samples were digested with 100 μg/mL of freshly prepared proteinase K (Gibco BRL, Gaithersburg, Md) at 56°C for 4 hours. After digestion, samples were centrifuged at 1000g for 5 minutes. Supernatants were collected and mixed well with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). After centrifugation at 14,000g for 15 minutes, DNA in the upper aqueous phase was incubated with 10 μg/mL of DNase free RNase A (Gibco BRL) at 37°C for 30 minutes to eliminate RNA. DNA was re-extracted with phenol/chloroform/isoamyl alcohol and precipitated at −80°C for 30 minutes in isopropanol followed by centrifugation at 14,000g at 4°C for 20 minutes. The resulting DNA pellet was washed with 75% ethanol and dissolved in Tris-EDTA buffer. The DNA concentration was determined at 260 nm by spectrophotometry. The purified DNA samples were subjected to DNA fragmentation analysis according to the manufacturer’s instructions (Clontech). DNA samples (0.5 μg) were ligated with dephosphorylated adaptors (5’-TGCGGTGAGAGG-3’, 5’-AGCACTCTCGAGGCCTCTCACCCGGA-3’) in the presence of T4 DNA ligase at 16°C for 16 hours. The adaptor-ligated DNA (100 ng) was amplified by polymerase chain reaction (PCR) with the use of Advantage cDNA Polymerase Mix (Clontech). The PCR products were visualized on a 1.2% agarose gel with ethidium bromide.

Apoptosis—enzyme immunoassay for cytoplasmic histone–associated DNA fragments. Apoptosis was quantified by using a photometric enzyme immunoassay (cell death detection ELISA; Boehringer Ingelheim, Burlington, Canada) to measure cytoplasmic histone–associated DNA fragments (mono- and oligonucleotides) as we previously described. Briefly, liver homogenate was centrifuged at 20,000g for 10 minutes at 4°C. The protein content of the supernatant was determined by Bradford assay. A known amount of protein (40 μg) was diluted to 100 μL for each sample and was added in duplicate to a microtiter plate coated with antihistone antibody. After 90 minutes of incubation, the samples were washed, anti-DNA peroxidase was added to each well, and the plate was washed again and 2,2’-azino-di-3-ethylbenzthiazoline sulfonate was added for color development. Absorbance was measured at 405 nm.

Apoptosis—caspase-3 activity. Caspase-3 activity was measured with the use of a caspase-3 fluorescent assay kit (BIOMOL; Research Laboratories, Plymouth Meeting, Pa) similarly to our previous...
report but with modifications. This assay is based on a fluorescence intensity of 7-amino-4-methyl-coumarin after cleavage by caspase-3 from the C-terminus of the peptide substrate. Briefly, frozen liver samples were homogenized in lysis buffer (50 mmol/L HEPES, pH 7.4, 0.1% CHAPS, 1 mmol/L DTT, 0.1 mmol/L EDTA), and the protein concentration was determined by Bradford assay.

Samples in duplicate (200 µL g protein) were incubated with caspase-3 substrate (Ac-DEVD-AMC) or with substrate plus inhibitor (AC-DEVD-CHO) at room temperature for 3 hours. Caspase-3 activity was quantified with the use of a fluorescent spectrophotometer (excitation at 355 nm; emission at 460 nm) and normalized by using inhibitor-treated samples as background.

**Heme oxygenase activity.** HO activity was measured in the liver microsomes as described previously. Briefly, microsomes were prepared from harvested tissues and subsequently added to a reaction mixture containing potassium phosphate buffer (0.1M KPBS, pH 7.4, hemin 25 µmol/L, (Porphrin Products Inc, Logan, Utah), and mouse liver cytosol (as a source of biliverdin reductase) prepared from 105,000 g supernatant. The reaction was initiated by addition of NADPH (0.4 mmol/L) to the samples, while the same volume of 0.1M KPBS was added to the blanks. The reaction was conducted in duplicate, in the dark, in a shaker water bath at 37°C for 30 minutes, and terminated by placing the samples on ice. Bilirubin concentration was calculated on the basis of the difference in absorbance at 470 and 530 nm, by using an extinction coefficient of 40 mmol/L cm⁻¹. HO activity was then expressed as picomoles of bilirubin produced per hour per milligram of protein, as determined by Bradford assay.

**Statistics.** Significance was determined by using standard analysis of variance with the Student-Newman-Keuls post hoc comparison (P < .05). All data was expressed as mean ± standard error.

**RESULTS**

Remote liver injury was quantified after 1 hour of bilateral hind limb ischemia and after 3 hours (early phase, included for comparison), 6 hours, or 24 hours (progressive phase) of reperfusion. As illustrated in Fig 1, a 4-fold increase (P < .05) in HO activity was measured 3 hours after I/R. This increase persisted throughout the 24-hour protocol. Chromium mesoporphrin achieved 60% inhibition of HO activity at 6 and 24 hours of reperfusion (Fig 1). The level of HO inhibition achieved in this study did not decrease HO activity below the level found in sham animals. Although a robust survival study was not performed, we observed that administration of CrMP to animals subjected to I/R resulted in 25% mortality at 24 hours of reperfusion (n = 8), whereas the mortality in untreated animals was 0% (n = 6). Mortality in a separate group of time-matched sham animals treated with CrMP was 0% (n = 6).

Hepatocellular injury, as measured by serum ALT, was initially increased 6-fold (P < .05) after hind limb ischemia and 3 hours of reperfusion, as compared with sham (Fig 2). Serum ALT was further increased to 8-fold (P < .05) at 6 hours of reperfusion. There was no further increase in serum ALT at 24 hours. Inhibition of HO activity by administration of CrMP resulted in the exacerbation of hepatocellular injury over 24 hours. Although CrMP did not affect the level of serum ALT after ischemia and 6 hours of reperfusion, there was a 14-fold increase in ALT at 24 hours in CrMP-treated animals. Administration of CrMP had no effect on serum ALT levels in sham animals (data not shown).

The intravital dye propidium iodide labels the nuclei of lethally damaged cells (predominantly via necrosis but also via end-stage apoptosis). The number of such labeled hepatocyte nuclei (identified morphologically) increased 3-fold (P < .05) after hind limb ischemia and 3 hours of reperfusion (Fig 3), with no further increase at later times suggesting hepatocyte death did not progress after the initial injury. Although CrMP had no effect on propidium iodide labeling at 6 hours.
compared with untreated animals, such treatment resulted in a 1.5 fold increase in labeling at 24 hours (P < .05). Administration of CrMP had no effect on propidium iodide staining in sham animals (data not shown). These data suggest that HO induction was an important protective mechanism limiting the progression of hepatocyte death during the later time points (up to 24 hours) after limb ischemia/reperfusion.

To determine whether apoptosis was involved in liver injury after limb I/R, we analyzed apoptosis by 3 methods. Genomic DNA fragmentation was measured by ligation-mediated PCR ladder assay, a very sensitive method for qualitative assessment of apoptosis. A representative gel is depicted in Fig 4. Fragmentation of DNA in multiples of approximately 200 bp, which is characteristic of apoptosis, was observed after 6 hours of reperfusion. No DNA fragmentation was detected at 24 hours after reperfusion, suggesting a transient increase in apoptotic cell death. Administration of CrMP resulted in high levels of DNA fragmentation at 6 and 24 hours post-reperfusion.

The level of apoptosis was quantified with the use of a cell death detection ELISA assay. A 28% increase in apoptosis (P < .05) was measured 6 hours after limb I/R compared with sham (Fig 5). The level of apoptosis measured at 24 hours was not different from that measured in sham animals, suggesting a transient increase in apoptosis after hind limb ischemia and 6 hours of reperfusion. Administration of CrMP resulted in a 2-fold increase in apoptotic cell death (P < .05) at both 6 and 24 hours of reperfusion. There was no significant difference in the level of apoptotic cell death at 24 hours compared with 6 hours in CrMP-treated animals.

Apoptosis was further confirmed by measurement of caspase-3 activity, a hallmark indicator of apoptosis. Activation of the caspase cascade occurs early during apoptosis and is known to precede increased DNA fragmentation. Accordingly,
caspase-3 activity was increased 3-fold \((P < .05)\) after ischemia and 3 hours of reperfusion (Fig 6), preceding the increased DNA fragmentation at 6 hours. Caspase-3 activity returned to sham levels at 6 and 24 hours of reperfusion. Administration of CrMP resulted in a 4-fold increase \((P < .05)\) in caspase-3 activity at 6 hours of reperfusion. However, caspase-3 activity returned to sham levels by 24 hours of reperfusion even in CrMP-treated animals. These data provide strong evidence in support of a transient increase in apoptotic activity and of a role for HO in inhibiting the duration and magnitude of this transient increase.

Hepatic microvascular perfusion was characterized and expressed as the percentage of continuously perfused sinusoids. The percentage of intermittently perfused sinusoids and increased percentage of nonperfused sinusoids at 3 hours of reperfusion. These deficits persisted at 6 hours, but perfusion was completely restored at 24 hours. Administration of CrMP resulted in abrogation of microvascular perfusion over 24 hours. *Denotes significant difference from sham; \(P < .05\). #Denotes significant difference from untreated animals at equivalent reperfusion time; \(P < .05\).
Administration of CrMP had no effect on microvascular perfusion in sham animals (data not shown).

**DISCUSSION**

It has been widely accepted that injury to remote organs can be caused by the systemic release of cytokines, procoagulants, cell debris, and other proinflammatory mediators upon reperfusion of ischemic limb(s). Studies using tourniquet shock models demonstrated important roles for leukocyte activation and sequestration during the progressive phase of liver injury. However, fluid resuscitation in our model maintained normotension throughout the protocol, thereby eliminating shock as a confounding variable. With this normotensive model, our previous studies provided direct evidence that remote liver injury occurred early and progressed over 3 hours after bilateral hind limb I/R. More recently, we established a protective role for endogenous HO in the initiating phase of remote liver injury with investigations limited to 1, 1.5, and 3 hours after bilateral hind limb I/R. At these early postreperfusion times, inhibition of endogenous HO exacerbated perfusion deficits, hepatocellular injury, and leukocyte recruitment. However it is important to note that in spite of such protection, endogenous HO activity during the first 3 hours after limb I/R was insufficient to completely prevent the onset and progression of liver injury.

In the present study, we extended our investigations to determine if HO activity was effective in limiting the progression of liver injury during prolonged periods after limb I/R (ie, through 24 hours). Contrary to indications from our previous studies, we show that the liver demonstrated recovery rather than progressive injury, as evidenced by restoration of microvascular perfusion and lack of increased hepatocyte death. The present study demonstrated that the elevated HO activity shown to occur within 3 hours of limb reperfusion continued for at least 24 hours. Administration of the selective competitive inhibitor CrMP to reduce HO activity to sham levels resulted in a progressive decline in hepatic perfusion, as well as increased hepatocellular injury and death.

Propidium iodide enters cells with severely compromised membranes, labeling predominantly those cells that have died or will die via necrosis and end-stage apoptosis. In contrast to necrotic cell death, apoptosis is a process by which cells undergo inducible cellular suicide in response to specific stimuli. Because each method of assessing apoptosis may have its inherent limitations, different methods were used. The data presented in the present study suggest a transient increase in apoptotic activity after hind limb ischemia and 6 hours of reperfusion in untreated animals. During the inhibition of HO activity via administration of CrMP, this transient increase in apoptosis appeared to be prolonged, as well as increased in magnitude. Taken together such data implicate endogenous HO as a mechanism limiting apoptosis in the liver after limb I/R.

Many of the mechanisms by which HO confers protection have been elucidated and are, in part, attributed to the products of heme catabolism. Reduced iron (Fe2+) released directly into the endoplasmic reticulum is sequestered into the intracellular iron storage protein ferritin, which is increased in tandem with HO-1 induction. The intracellular capture of free iron may be a contributor to the antiapoptotic effects of HO, although carbon monoxide (CO) is also implicated as an antiapoptotic mechanism. Bilirubin and CO reduce oxidative stress by scavenging reactive oxygen species. Carbon monoxide is a potent vasodilator and is known to regulate sinusoidal tone both by activation of soluble guanylate cyclase and through direct action on Ito cells. In addition, CO can inhibit platelet aggregation and has been shown to inhibit the proinflammatory cytokines TNF-α, interleukin-1β, and macrophage inflammatory protein-1β. Although the precise mechanism(s) remain to be determined, HO activity has been shown to provide anti-inflammatory benefits by downregulating ICAM-1, P-selectin, and E-selectin, as well as by inhibiting TNF-α-induced apoptosis in vitro. Although much progress has been made in the study of HO as an anti-inflammatory, antioxidant, and antiapoptotic agent, the mechanisms of these actions require further study.

**CONCLUSION**

Heme oxygenase activity in the liver was significantly increased after limb I/R. Inhibition of HO to levels consistent with sham increased microvascular perfusion deficits, hepatocellular injury, and the duration and magnitude of the transient increase in apoptotic cell death. We believe this study is novel in that it provides the first direct evidence establishing (1) the ability of the liver to recover from an otherwise progressive deterioration after limb I/R, (2) the importance of HO activity as at least 1 mechanism leading to such liver recovery, and (3) the ability of HO to modulate apoptosis in the liver after limb I/R.
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REFERENCES


